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(57) Abstract

The present invention provides a silage composition supplemented with endo-xylanase activity. According to the present invention, the addition of endo-xylanase to fodder produces a silage having a significant increase in the amount of water soluble carbohydrates for use by lactic acid bacteria or as nutrients for the animal ingesting the silage; and increased fermentative production of lactic acid and acetic acid by lactic acid bacteria and the consequent lowering of pH which serves to preserve the silage composition. Also provided are silage compositions which further contain an inoculum of a lactic acid bacteria.

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# ENZYMATIC TREATMENT OF SILAGE

The present invention relates to the field of agriculture, more specifically, the present invention relates to an improvement in the enzymatic treatment of fodder silage.

## Background of the Invention

Improvements in silage technology have, next to natural and artificial drying, made ensiling the most widely applied technology for the preservation of forage. It has been estimated that in western Europe, 60% (approximately 77 million tons dry matter) of the forage preserved for winter is ensiled. In the U.S., approximately 80 million tons (dry matter) are ensiled per year. Ensiling is also widely used in eastern Europe and Canada.

However, effluent production and nutrient losses in silage caused by fermentation (especially butyric acid fermentation) and aerobic deterioration have become increasingly problematic with the growing demand for higher efficiency in animal livestock production. These problems often depend on factors such as the type of crop, climactic conditions and available technology. Recently introduced production limits for dairy farming and, in some countries, environmental legislation indirectly restricting the type and amounts of nutrients used for livestock production emphasize the need to feed high quality silages (Spoelstra, S.F. (1991) In: Proceedings of the EGF Conference - "Forage Conservation Towards 2000"; Braunschweig, Germany).

Grasses (such as rye-grasses, etc.), cereals (such as maize, sorghum, wheat, etc.) and legumes (such as alfalfa, clover, etc.) are the primary crops used as fodder for ensiling. Beet tops and sugar beet pulp are also used in relatively smaller amounts.

Maize ensiles well, but is susceptible to aerobic deterioration. Grass, mainly perennial rye-grass, is often heavily fertilized and has, as a result, a low ratio of water soluble carbohydrates to buffering capacity making it susceptible, after ensiling, to butyric acid fermentation. Butyric acid fermentation leads to dry matter losses in the silage, thus lowering the nutritional value and consequently, livestock production efficiency.

To avoid undesirable butyric acid fermentation, the grass is normally wilted in the field to, on average, 500 g DM (dry matter) per kg. Alternatively, at DM contents below 350 g DM per kg, additives such as enzymes are applied.

Continuing research efforts have been undertaken to study whether silage preservation is enhanced and silage intake and digestibility is improved by the addition of cell wall degrading enzymes, cellulases and hemicellulases, in particular.

The actions of cellulases and hemicellulases liberate water soluble carbohydrates from the structural polysaccharides of the plant cell wall, thus providing substrates for the production of lactic acid via fermentation by the lactic acid bacteria present in the silage. These enzymes are probably also responsible for the biolytic disruption of the plant cell walls, releasing further water soluble carbohydrates and other materials from within the cell for use by the lactic acid bacteria. In addition, the pre-digestion of the plant cell walls during the storage of the silage may lead to their being more rapidly degraded in the rumen and thus enhance digestibility and moreover may provide increased amounts of liberated carbohydrates for added nutritional value for the animal ingesting the thus-treated silage.

The production of lactic acid (as well as acetic acid) by lactic acid bacteria present in the silage is also responsible for the lowering of the pH of the silage. The lowering of the pH, generally decreasing to about pH 4.5 and

may be as low as about pH 4, creates an unfavorable environment for the growth of yeasts, as well as many other undesired microbes such as butyric acid-producing microorganisms, thus preserving the silage and maintaining its nutritional content.

Additionally, the lactic acid bacteria also serve as probiotics, beneficially enhancing the intestinal flora of the animal ingesting the silage.

Most, if not all enzyme preparations investigated for use in silage are rather crude fermentation products from fungi. These enzyme preparations contain many different enzyme activities.

For example, a cellulase preparation, obtained from a Penicillium verruculosum mutant strain, is disclosed in East German patent publication DD-278359 (published May 2, 1990) to be applicable for partial or total hydrolysis of cellulose and hemicellulose in industrial processes such as the production of silage, inter alia. The cellulase preparation is purported to contain high cellulolytic activity combined with beta-glucosidase, xylanase and amylase activity.

A probiotic inoculum for silage comprising lactic acid bacteria transformed with exogenous DNA encoding an enzyme capable of degrading polysaccharides and oligosaccharides in a silage crop to provide a source of water soluble carbohydrates for the bacteria themselves is described in PCT Application WO 89/01970 (published March 9, 1989).

## Summary of the Invention

According to the present invention, it has been found that endo-xylanase activity, of the many enzymatic activities present in enzymatic preparations used to ensile fodder, is particularly responsible for the release of water soluble carbohydrates, which through the fermentative action of lactic acid bacteria, leads to the preservation and improved nutritional quality of the silage. Furthermore, it

has been found that supplementing fodder to be ensiled with sufficient amounts of endo-xylanase, either with or without the presence of other enzymatic activities, provides optimal preservation and nutritional quality of the thus-treated silage. It has also been observed that animals ingest greater amounts of silage preserved in this manner, as opposed to silage which has deteriorated. These factors all lead to an enhancement in the efficiency of livestock production.

The present invention thus provides a silage composition supplemented with an amount of endo-xylanase activity effective in releasing water soluble carbohydrates from the ensiled plant cell material (fodder), thus enhancing the production of lactic acid and acetic acid by lactic acid bacteria, which in turn improves the preservation and nutritional quality of the silage. The endo-xylanase is preferably added in a form which is essentially free of other enzymatic activity.

As the fodder to be ensiled does not always contain sufficient amounts of lactic acid bacteria to achieve proper ensiling, the present invention also provides silage compositions which further contain an inoculum of a lactic acid bacteria, included as a supplement in an amount sufficient to assist the preservation of the silage and to act as probiotics for the enhancement of the intestinal flora of the animal ingesting the thus-treated silage composition.

It is also an object of the present invention to provide a method of preserving silage characterized in that the silage is supplemented with an amount of endo-xylanase activity effective in increasing the amount of water soluble carbohydrates present in the silage for use in the production of lactic acid and acetic acid by lactic acid bacteria.

It is a further object of the present invention to provide a method for the improvement of the nutritional

value of silage by supplementing the silage with an amount of endo-xylanase activity effective in releasing water soluble carbohydrates from the ensiled plant cell material for utilization by the animal ingesting the silage. The endo-xylanase present in the silage, also provides a supplement of an essential enzyme for the animal itself.

It is yet another object of the present invention to provide a method for improving the in vivo (dry matter) digestibility of silage in animals wherein the animal is fed a diet consisting at least in part of a silage which has been supplemented with an amount of endo-xylanase activity effective in at least partially degrading, and thus pre-digesting, plant cell walls.

## Brief Description of the Figure

Figure 1: HPLC elution profile of a culture filtrate obtained from Aspergillus niger DS16813 (CBS 323.90). This strain was later reclassified as more likely belonging to the species Aspergillus tubigensis.

## Detailed Description of the Invention

According to the present invention, fodder to be ensiled is supplemented with an amount of endo-xylanase which is effective in producing a silage having a significant increase in the amount of water soluble carbohydrates for use by the lactic acid bacteria or as nutrients for the animal ingesting the silage; and improved qualities such as increased fermentative production of lactic acid and acetic acid (lactic acid being produced in greater quantities relative to acetic acid) by (probiotic) lactic acid bacteria and the consequent lowering of pH which serves to preserve the silage composition and its nutritional value, these factors contributing to the improvement of livestock production efficiency. Until the present invention, endo-xylanases were not known to be

specifically responsible for such a significant role in the production of improved silages. Nor was it known that the addition of an endo-xylanase in a form which is substantially free of other enzymatic activity (especially other cell wall degrading enzymatic activity) to fodder to be ensiled, would lead to obtaining a silage which is not only better preserved, but has improved nutritional quality as well.

Silage compositions, according to the present invention, preferably contain more than 50,000 units endo-xylanase activity per kg (fresh weight) fodder. The endo-xylanase optimally has a pH optimum in the range of pH 3.5 to pH 6.0 in order to ensure that maximum enzyme efficacy is obtained at the acidic pH conditions which exist in the process of ensiling fodder. As stated above, the endo-xylanase is preferably added in a form which is substantially free of other enzymatic activity in general, and cell wall degrading enzymatic activity in particular.

The supplementation of the fodder to be ensiled with endo-xylanase activity which is substantially free of other enzymatic activities is also commercially attractive as a more cost-effective alternative to the use of enzyme mixtures wherein secondary enzyme activities often must be supplemented to obtain the desired effect. The phrase "substantially free of other enzymes", as defined in context of the present invention, intends that the majority (i.e. at least 50% per weight, preferably at least 80% per weight) of the total protein found in the enzymatic preparation is endo-xylanase.

For example, the endo-xylanase activity may be produced in large quantities using recombinant DNA techniques, such as described in European Patent Application no. 90202020.5 (filed July 24, 1990), the disclosure of which is hereby incorporated by reference. Alternatively, the production of endo-xylanases may be enhanced by adjusting the fermentation conditions of the endo-xylanase-

producing microorganism such as the use of an inducing media containing xylan.

Endo-xylanases, for use in the context of the present invention, are preferably obtained from a filamentous fungus, more preferably from a filamentous fungus selected from the genera consisting of Aspergillus, Disporotrichum, Penicillium, Neurospora, Fusarium and Trichoderma, and most preferably obtained from a filamentous fungus selected from the species consisting of Aspergillus niger, Aspergillus awamori, Aspergillus aculeatus, Aspergillus tubigensis, Disporotrichum dimorphosporum and Trichoderma reesei.

An endo-xylanase to be used in the present invention may be identified via assay methods not critical to the present invention, such as a spot test assay. According to this method, a filtrate obtained from the culturing of a microorganism induced (e.g. with oat spelt xylan) to produce an endo-xylanase may be tested for the presence of endo-xylanase activity. Drops of the elution fractions are placed individually onto an agar film containing a citrate-phosphate buffer (see Example 1.1, below) and oat spelt xylan. The film is then incubated. If endo-xylanase activity is present, the locations of the individual drops on the agar film are visibly clear.

Endo-xylanase activity may also be identified by subjecting a xylan-containing solution to an enzyme solution suspected of containing endo-xylanase activity and spectrophotometrically analyzing the reducing sugars by the method as described by Leathers, T.D. et al. (1984) Biotechnol. Bioeng. Symp., 14, 225.

A unit of endo-xylanase activity is herein defined as the amount mol) of xylose equivalents liberated per minute per mg prc in at a temperature of 39°C and at pH 3.5. Protein determination was performed according to the method as described by Bradford, M.M. (1976) Anal. Biochem., 72, 248. Bovine immuno-gamma-globulin (BIgG) was used as the protein standard.

Once identified, an endo-xylanase-producing organism may be fermented under conditions conducive to the production of endo-xylanase and the desired enzymatic activity may be isolated from the culture filtrate of the production organism and, if desired, purified by conventional means not critical to the present invention. For example, affinity and/or ion exchange chromatography techniques may advantageously be used for the preparative purification of an endo-xylanase from a culture filtrate.

Another embodiment of the present invention also envisions the supplementation of commercial enzyme preparations with endo-xylanase activity. Preferably, an amount of endo-xylanase is added to provide a total endo-xylanase activity in the final mixture of more than 50,000 units/kg fresh weight fodder.

In yet another embodiment of the present invention silage compositions are provided which further contain an inoculum of a lactic acid bacteria, included as a supplement to assist in the preservation of the silage. The lactic acid bacteria is preferably selected from the group consisting of the genera Lactobacillus, Enterococcus, Lactococcus, Pediococcus and Leuconostoc. According to the present invention, the most preferred lactic acid bacteria is selected from the group consisting of Lactobacillus plantarum, Lactobacillus acidophilus, Lactobacillus brevis, Lactobacillus bulgaricus, Lactobacillus fermentum and Lactobacillus lactis.

The supplementation of endo-xylanase to silage, according to the present invention may be applied to all ensilable plant material such as grasses, cereals and legumes, as known to those skilled in the art. The fodder normally, however, will have a dry matter (DM) content of below 350 g DM per kg fodder (fresh weight) to allow the enzyme free access to the plant cell material.

The following examples are provided so as to give those of ordinary skill in the art a complete disclosure and

description of how to make and use the invention and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperature, pH, etc.) but some experimental errors and deviation should be accounted for. Unless indicated otherwise, temperature is in degrees Celsius and pressure is at or near atmospheric.

Example 1

Purification of Aspergillus tubigensis endo-xylanase.

A culture filtrate was obtained by the culturing of Aspergillus niger DS16813 (CBS 323.90, deposited at the Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands on July 20, 1990 - later reclassified as more likely belonging to the species A. tubigensis; Kusters-van Someren et al. (1991) Curr. Genet. **19**, 21) in a medium containing (per liter): 30 g oat spelt xylan (Sigma); 7.5 g $NH_4NO_3$, 0.5 g KCl, 0.5 g $MgSO_4$, 15 g $KH_2PO_4$, and 0.5 g yeast extract (pH 6.0). The culture filtrate was concentrated to a volume of approximately 35 ml which was then was ultrafiltered on a Diaflo PM 10 filter in a 50 ml Amicon module to remove salts.

The supernatant was then concentrated to a volume of 10 ml and the retentate was washed twice with 25 ml 25 mM Tris-HCl buffer (pH 7.0). After washing, the retentate volume was brought to 25 ml.

This retentate was injected in 1 ml quantities onto a Syn Chropak AX 300 column (dimensions 10 x 250 mm) and eluted in the following HPLC regime:

elution rate: 2 ml/min.
elution buffer A: 25 mM Tris-HCl pH 7.0
elution buffer B: 25 mM Tris-HCl pH 7.0 + 1 M NaCl
elution gradient:

| time (min) | %A | %B |
|---|---|---|
| 0 | 99 | 1 |
| 12 | 97 | 3 |
| 30 | 80 | 20 |
| 50 | 50 | 50 |
| 70 | 0 | 100 |
| 90 | 0 | 100 |
| 95 | 99 | 1 |

Fractions of 1 ml each were collected. Detection of the eluted protein was performed by continuous measurement of the UV absorption at 280 nm. The elution profile is shown in Figure 1.

The fractions were tested for the presence of endo-xylanase activity by a spot test. This spot test consists of adding 12 ml citrate-phosphate buffer (prepared by mixing 900 ml 0.2 M $Na_2HPO_4$ and 125 ml 0.5 M citric acid, followed by an adjustment of the pH of the solution to pH 5.6 using 0.5 M citric acid or 0.2 M $Na_2HPO_4$) containing 0.5% oat spelt xylan (Sigma) to 180 mg agar (Difco) and heating the mixture to 100°C to dissolve the agar. After cooling to 60°C, the agar mixture is poured evenly onto Agarose gel-bond film. Drops of the elution fractions are placed individually onto the film and incubated for 30 min. at 30°C. If endo-xylanase activity is present, the location of the individual drops on the agar film is clear.

Total xylanase activity in the collected fractions was quantitatively determined by measuring the amount of reducing sugars produced over a predetermined time period in the microassay as described by Leathers et al. (1984), using oat spelt xylan in 50 mM sodium acetate at pH 5.0 as a substrate. Activity units are also as defined by Leathers (supra).

Exo-xylanase activity in the eluted fractions was determined by the method described by Poutanen and Puls (1988), using p-nitro-phenyl-ß-D-xylopyranoside (0.3 mM, Sigma) as a substrate at pH 5.0 and 30°C.

The spot test revealed that the elution fractions corresponding to peaks B, F and K (see Figure 1) contain endo-xylanase activity. The total xylanase assay showed activity in the elution fractions of peaks B, F, H and K. The elution fractions of peaks B and H were determined to contain exo-xylanase activity.

The elution fractions of peaks F (XYL2 protein) and K (XYL A protein) were further purified by repeated ion exchange chromatography.

Example 2

Perennial English rye-grass was mown, dried for 24 hours until reaching a dry matter (DM) content of about 25%, cut into lengths of 1-2 cm and ensiled in 1 liter laboratory silos and stored for 3 months at ambient temperature before analysis.

The enzyme preparation Cellulase ABG-7 was added at a concentration of 0.2 g of protein per kg fresh weight grass (equivalent to 3200 units of endo-xylanase activity per kg fodder; protein content determined using the method as described by Bradford, M.M. (supra)). Cellulase ABG-7 is a commercial enzyme preparation produced by Trichoderma reesei and is available under the name MAXAZYM® CL-2000 (IBIS, N.V., Rijswijk, The Netherlands). The enzymatic activities of this product are summarized in Table 1, below.

Table 1: Enzymatic activities of Cellulase ABG-7

| ACTIVITY | MEASURED PRODUCT | SPECIFIC ACTIVITY μmoles/min.Mg protein |
|---|---|---|
| CMC-ase | glucose equivalent | 0.79 |
| Avicelase | glucose equivalents | 0.11 |
| Endoxylanase | xylose equivalents | 16.0 |
| Exoxylanase | p-nitrophenol | 0.48 |
| Exoarabinase | p-nitrophenol | 0.51 |
| Acetylesterase | p-nitrophenol | 0.16 |
| Polygalacturonase | galactose equivalents | 3.56 |
| Glucuronidase | p-nitrophenol | 0.003 |
| α-Amylase | glucose | 0.25 |

The results of the silage experiment are presented in Table 2, below.

Table 2: Mean (N=3) composition of grass, control silage and enzyme-treated silage after incubation with Cellulase ABG-7

| | | | SILAGE | |
|---|---|---|---|---|
| | | GRASS | CONTROL | CELLULASE ABG-7 |
| Dry matter (DM) | (g/kg) | 273 | 269 | 259 * |
| Ash | (g/kg DM) | 124 | 124 | 136 * |
| Lactic acid | (g/kg DM) | - | 45.5 | 59.4* |
| Ethanol | (g/kg DM) | - | 7.33 | 19.1* |
| Acetic acid | (g/kg DM) | - | 46.9 | 82.8* |
| Crude fiber | (g/kg DM) | 258 | 262 | 194 * |
| Neutral Detergent Fiber | (g/kg DM) | 523 | 446 | 351 * |
| Acid Detergent Fiber | (g/kg DM) | 279 | 281 | 204 * |
| Acid Detergent Lignin | (g/kg DM) | 17.5 | 15.9 | 18.5* |
| Weight loss | (g/kg DM) | - | 49.9 | 76.3* |
| $NH_3$-N/total N | (wt% N-total) | - | 26 | 20 * |
| pH | | - | 5.42 | 4.72 |

* statistically different from the control.
$P < 0.05$ (Student T-test)

Cellulase ABG-7 hydrolysed a large proportion of cell wall constituents to fermentable carbohydrates. This resulted in increased silage quality as characterized by the liberation of water soluble carbohydrates. This is evidenced by the increased lactic acid concentration and lower crude fiber content, lower Neutral Detergent Fibre content, lower Acid Detergent Fiber content, as well as lower pH and ammonia values in comparison to the control.

Example 3

Perennial English rye-grass was prepared as described in Example 2, above, and ensiled in 1 liter laboratory silos and stored for 3 months at ambient temperature before analysis.

The enzyme Cellulase ABG-7 (see Table 1, above) was added at concentrations of 0.005 g of protein (80 units endo-xylanase activity) per kg fresh weight grass; 0.025 g of protein (400 units endo-xylanase activity) per kg fresh weight grass; 0.050 g of protein (800 units endo-xylanase activity) per kg fresh weight grass; 0.075 g of protein (1200 units endo-xylanase activity) per kg of fresh weight grass (all protein content determined using the method as described by Bradford, M.M. (supra)).

The results are presented in Table 3.

Table 3 : Mean (N=3) composition of grass and silages.

| | | GRASS | CONTROL SILAGE | SILAGE | | | |
|---|---|---|---|---|---|---|---|
| | | | | CELLULASE ABG-7 (g protein/kg grass) | | | |
| | | | | 0.005 | 0.025 | 0.050 | 0.075 |
| Dry matter (DM) | (g/kg) | 233 | 223 | 223 | 221 * | 219 * | 219 * |
| Ash | (g/kg DM) | 111 | | | | | |
| Lactic acid | (g/kg DM) | - | 117 | 102 | 116 | 126 | 120 |
| Ethanol | (g/kg DM) | - | 5.73 | 4.22* | 4.44* | 4.97* | 4.95* |
| Acetic acid | (g/kg DM) | - | 33.7 | 26.5 * | 20.0 * | 32.6 | 34.5 |
| Crude fibre | (g/kg DM) | 256 | 249 | 252 | 247 | 242 | 233 * |
| NDF | (g/kg DM) | 505 | 428 | 430 | 427 | 413 * | 403 * |
| ADF | (g/kg DM) | 275 | 268 | 266 | 261 | 257 * | 254 * |
| ADL | (g/kg DM) | 19.7 | 20.8 | 20.4 | 21.8 | 20.8 | 20.7 |
| Weight loss | (g/kg DM) | - | 39.9 | 26.9 * | 25.8 * | 34.7 | 36.8 |
| $NH_3$-N/total N | (wt % N-total) | - | 16 | 14 * | 11 * | 14 * | 15 |
| pH | | - | 4.25 | 4.09* | 3.93* | 4.10* | 4.11* |

* statistically different from the control. $P < 0.05$ (Student T-test)

Example 4

Perennial English rye-grass was prepared as described in Example 2, above, and ensiled in 1 liter laboratory silos and stored for 3 months at ambient temperature before analysis.

In this experiment, a purified endoxylanase from Aspergillus tubigensis (specific activity 5000 units endo-xylanase activity per kg fodder (Bradford protein determination method, supra); see Example 1) was combined with the Cellulase ABG-7 preparation to increase the endoxylanase activity in the preparation and added to the grass. The endoxylanase concentration in this combined enzyme product added to the grass was 0.050 g of protein (250,000 units endo-xylanase activity) per kg (fresh weight) grass and the Cellulase ABG-7 concentration added was 0.200 g of protein (3200 units endo-xylanase activity) per kg (fresh weight) grass (protein contents determined using the method as described by Bradford, M.M. (supra)).

The results are presented in Table 4.

Table 4 : Mean (N=3) composition of grass and silages.

| | | | SILAGE | | |
|---|---|---|---|---|---|
| | | GRASS | CONTROL | CELLULASE ABG-7 | CELLULASE ENDOXY-LANASE |
| Dry matter (DM) | (g/kg) | 273 | 269 | 259 * | 259 * |
| Ash | (g/kg DM) | 124 | 124 | 136 * | 133 * |
| Lactic acid | (g/kg DM) | - | 45.5 | 59.4 * | 101 * |
| Ethanol | (g/kg DM) | - | 7.33 | 19.1 * | 7.81* |
| Acetic acid | (g/kg DM) | - | 46.9 | 82.8 * | 46.6 * |
| Crude fibre | (g/kg DM) | 258 | 262 | 194 * | 192 * |
| NDF | (g/kg DM) | 523 | 446 | 351 * | 344 * |
| ADF | (g/kg DM) | 279 | 281 | 204 * | 205 * |
| ADL | (g/kg DM) | 17.5 | 15.9 | 18.5 * | 17.3 |
| Weight loss | (g/kg DM) | - | 49.9 | 76.3 * | 46.4 |
| $NH_3$-N/total N | (wt % N-total) | - | 26 | 20 * | 20 * |
| pH | | - | 5.42 | 4.72* | 4.39* |

* statistically different from the control. $P < 0.05$

Surprisingly, the addition of endo-xylanase to the Cellulase ABG-7 preparation resulted in a much higher concentration of lactic acid in the ensiled grass and subsequently a much lower pH.

Moreover the fermentation of ethanol and acetic acid is suppressed as well as the weight loss due to the formation of $CO_2$ and volatile fatty acids.

Example 5

Perennial English rye-grass was prepared as described in Example 2, above, and ensiled in 1 liter laboratory silos and stored for 3 months at ambient temperature before analysis.

Purified endoxylanase was added to the grass in concentrations ranging of 0.010 g, 0.050 g and 0.200 g of protein per kg fresh weight fodder (Bradford protein determination method, supra).

The results are presented in Table 5.

Table 5 : Mean (N=3) composition of grass and silages.

| | | GRASS | CONTROL SILAGE | SILAGE ENDOXYLANASE (units/kg grass) 50,000 | 250,000 | 1 x $10^6$ |
|---|---|---|---|---|---|---|
| Dry matter (DM) | (g/kg) | 273 | 269 | 263 | 267 | 264 |
| Ash | (g/kg DM) | 124 | 124 | 126 | 127 * | 130 * |
| Lactic acid | (g/kg DM) | - | 45.5 | 50.0 | 67.4 * | 90.2 * |
| Ethanol | (g/kg DM) | - | 7.33 | 6.54 | 7.02 | 7.63 |
| Acetic acid | (g/kg DM) | - | 46.9 | 48.2 | 53.2 * | 55.9 * |
| Crude fibre | (g/kg DM) | 258 | 262 | 263 | 246 * | 219 * |
| NDF | (g/kg DM) | 523 | 446 | 438 | 417 * | 374 * |
| ADF | (g/kg DM) | 279 | 281 | 283 | 267 * | 236 * |
| ADL | (g/kg DM) | 17.5 | 15.9 | 16.8 | 16.8 | 16.7 |
| Weight loss | (g/kg DM) | - | 49.9 | 51.4 | 54.0 * | 53.2 * |
| $NH_3$-N/total N | (wt % N-total) | - | 26 | 27 | 27 | 27 |
| pH | | - | 5.42 | 5.23 | 5.03* | 4.70* |

* statistically different from the control. $P < 0.05$ (Student T-test)

The results presented in Table 5 clearly demonstrate the efficacy of the supplementation of endo-xylanase activity in the production of an improved silage. The data illustrate a substantial increase of lactic acid concentration in the ensiled grass with increasing amounts of supplemented endo-xylanase activity. A slight increase of acetic acid content was observed. This in addition to the increased lactic acid content account for the lower pH and thus a better preservation of the silage. No significant increase in ethanol production (from undesirable yeast fermentation) was observed. Moreover, the data show a significant decrease of cell wall biopolymer parameters (lower crude fiber content, lower Neutral Detergent Fibre content and lower Acid Detergent Fiber content in comparison to the control), resulting in an increase in fermentable sugars available for lactic acid bacteria and for an animal consuming an endo-xylanase treated silage. The pre-digestion of the cell wall material also results in the enhanced digestibility of the ensiled fodder.

Claims:

1. An endo-xylanase-containing silage composition containing more than 50,000 units endo-xylanase activity per kg (fresh weight) fodder.

2. The silage composition of claim 1, further characterized in that the endo-xylanase is obtained from a filamentous fungus.

3. The silage composition of claim 2, further characterized in that the endo-xylanase is obtained from a filamentous fungus selected from the genera consisting of Aspergillus, Disporotrichum, Penicillium, Neurospora, Fusarium and Trichoderma.

4. The silage composition of claim 3, further characterized in that the endo-xylanase is obtained from a filamentous fungus selected from the species consisting of Aspergillus niger, Aspergillus awamori, Aspergillus aculeatus, Aspergillus tubigensis, Disporotrichum dimorphosporum and Trichoderma reesei.

5. The silage composition of claim 1, further characterized in that the endo-xylanase has a pH optimum in the range of pH 3.5 to pH 6.0.

6. The silage composition of claim 1, further characterized in that it further contains an inoculum of a lactic acid bacteria.

7. The silage composition of claim 6, further characterized in that the lactic acid bacteria is selected from the group consisting of Lactobacillus plantarum, Lactobacillus acidophilus, Lactobacillus brevis, Lactobacillus bulgaricus, Lactobacillus fermentum and Lactobacillus lactis.

8. A silage composition according to any one of claims 1-7, further characterized in that the endo-xylanase is supplemented to the fodder in a form substantially free of other enzymes.

9. A method of preserving silage characterized in that the silage is supplemented with an amount of endo-xylanase activity effective in increasing the amount of water soluble carbohydrates present in the silage for use in the production of lactic acid and acetic acid by lactic acid bacteria.

10. A method for improving the in vivo digestibility of silage in animals wherein the animal is fed a diet containing silage which has been supplemented with an amount of endo-xylanase activity effective in at least partially degrading plant cell walls.

11. A method for the improvement of the nutritional value of silage by supplementing the silage with an amount of endo-xylanase activity effective in releasing water soluble carbohydrates from the ensiled plant cell material for utilization by the animal ingesting the silage.

12. Use of an endo-xylanase in the preparation of silage.

13. A composition comprising at least 50 wt. % endo-xylanase for use in the treatment of silage.

14. A silage treated with more than 50,000 units endo-xylanase/kg (fresh weight) fodder.

15. An animal feedstuff composition comprising the silage of claim 14.

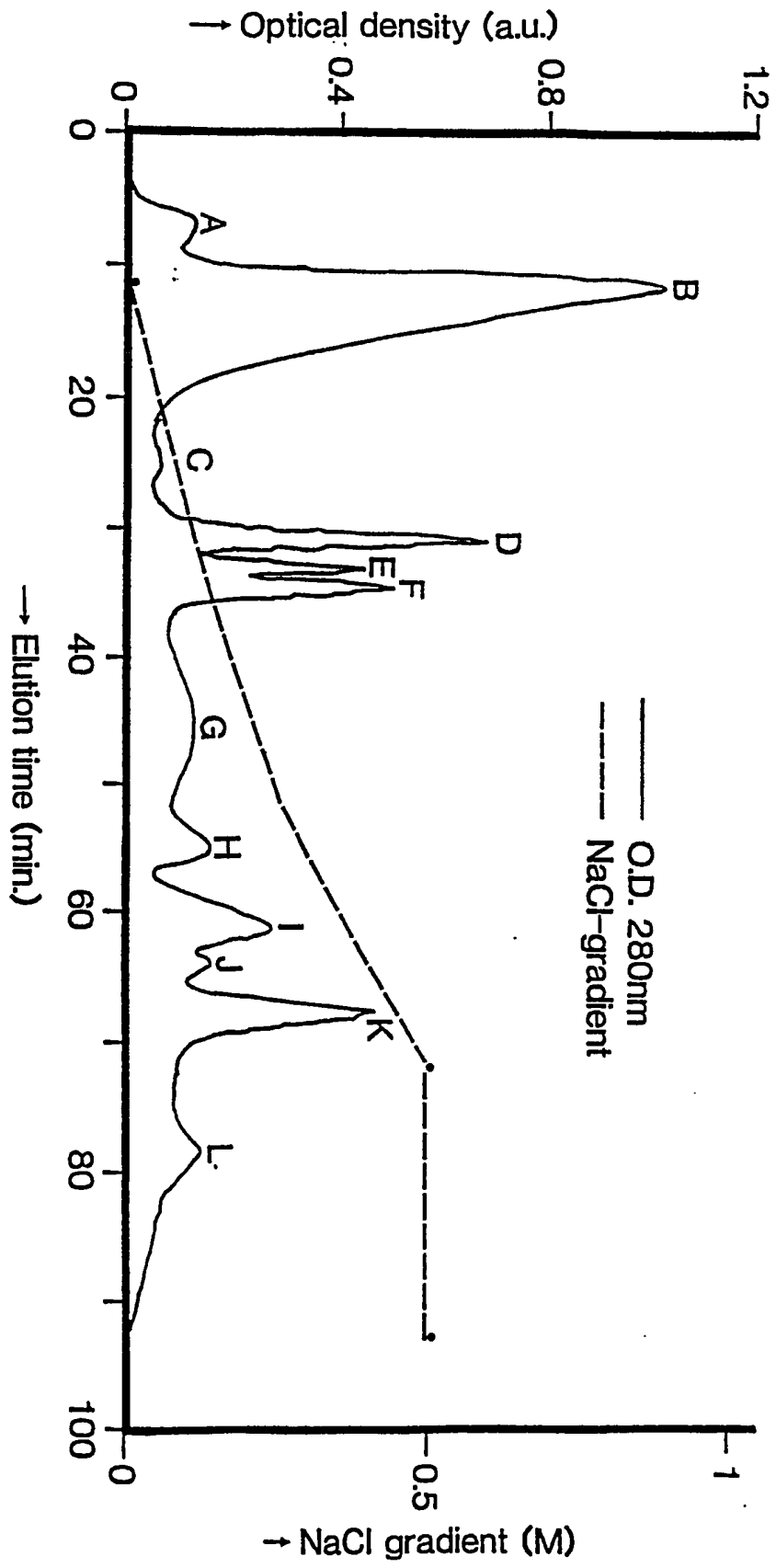
→ Optical density (a.u.)
0
0.4
0.8
1.2
0
20
40
60
80
100
A
B
C
D
E
F
G
H
I
J
K
L
→ Elution time (min.)
O.D. 280nm
NaCl-gradient
0
0.5
1
→ NaCl gradient (M)

Figure 1

# INTERNATIONAL SEARCH REPORT

International Application No PCT/NL 91/00136

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A23K3/03 ; C12N9/24 ; A23K1/165

## II. FIELDS SEARCHED

Minimum Documentation Searched[7]

| Classification System | Classification Symbols |
|---|---|
| Int.Cl. 5 | A23K ; C12N |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

## III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

| Category ° | Citation of Document, [11] with indication, where appropriate, of the relevant passages [12] | Relevant to Claim No.[13] |
|---|---|---|
| X | CHEMICAL ABSTRACTS, vol. 87, no. 22, November 28, 1977, Columbus, Ohio, US; abstract no. 182914, SILVERS V. S. ET AL 'Use of hydrolytic enzyme preparations during ensilage of corn cobs ' page 484 ; | 9,12 |
| A | see abstract | 1-8, 10-11, 13-15 |

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° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 17 SEPTEMBER 1991 | 24. 09. 91 |
| **International Searching Authority** | **Signature of Authorized Officer** |
| EUROPEAN PATENT OFFICE | LE CORNEC N.D.R. |

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category ° | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No. |
|---|---|---|
| X | CHEMICAL ABSTRACTS, vol. 94, no. 11, March 16, 1981, Columbus, Ohio, US; abstract no. 82520X, BODGAN S. D. ET AL 'Use of enzymic preparations for the ensilage of wheat straw. ' page 591 ; | 9,12 |
| A | see abstract | 1-8, 10-11, 13-15 |
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| A | WO,A,8 910 066 (CULTOROY) see claims; example 1 | 1-15 |

# ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

NL 9100136
SA 49974

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
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17/09/91

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO-A-8910066 | 02-11-89 | AU-A- 3449389 | 24-11-89 |
| | | EP-A- 0412094 | 13-02-91 |

EPO FORM P0479

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82